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## Review article

# Cathelicidins—Therapeutic antimicrobial and antitumor host defense peptides for oral diseases

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Received 5 March 2009; received in revised form 17 September 2010; accepted 24 November 2010

**KEYWORDS**

Host defense peptide;  
Cathelicidin peptide;  
Oral squamous cell carcinoma;  
Periodontal disease;  
Oral mucositis;  
Oral lichen planus

**Abstract** The oral epithelium functions as a mechanical and protective barrier to resist bacterial infection. Several types of host defense peptides (HDPs), including defensins, cathelicidins, and histatins, may have important roles in innate host defense. HDPs, which are mostly cationic and have amphipathic structures, provide non-specific, rapid defense against invading pathogens.

Human cationic antimicrobial protein 18 kDa (hCAP18/LL-37) is the only member of the cathelicidin family identified in humans thus far. Proteolytic processing releases LL-37 from its inactive precursor hCAP18/LL-37 to initiate its antimicrobial activity. As its name implies, LL-37 is made up of 37 amino acids. Various immune and epithelial cells secrete hCAP18/LL-37, and its level is altered in response to cariogenic, periodontal, congenital, inflammatory, and malignant diseases in the oral region. Human cathelicidin peptide, LL-37 exhibits antimicrobial activity against bacteria that cause oral pathological conditions, including cariogenic disease and periodontitis. Altered expression of hCAP18/LL-37 was observed in oral inflammatory lesions with and without microbial infection or oral cancer. Treatment with hCAP18/LL-37 may be useful in infectious, inflammatory, and cancerous diseases.

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**Contents**

Introduction . . . . .	68
The classification and the molecular structure of HDPs . . . . .	68
The molecular structure of human cathelicidin hCAP18/LL-37 . . . . .	69
Tissue distribution of hCAP18/LL-37 . . . . .	69
Various biological activities of the hCAP18/LL-37 peptide . . . . .	70
Mechanism of action and antimicrobial activity . . . . .	70
Peptide interaction with tumor cells and antitumor activity . . . . .	71

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Other biological activities	72
The involvement of hCAP18/LL-37 in the oral diseases	74
Caries and periodontitis	74
Periodontitis associated with congenital diseases	75
Inflammatory epithelial diseases	75
Malignant tumors	75
Therapeutic potential of HDPs (cathelicidin-based agents)	76
Conclusion	76
Acknowledgements	76
References	77

## Introduction

The human oral cavity is constantly exposed to a variety of microorganisms that could colonize and cause disease. Resistance to oral bacterial infection is offered by the oral mucosa membrane, which acts as a mechanical barrier, and saliva, which contains unique HDPs (also termed antimicrobial peptides) and increases mechanical action. Additionally, the oral mucosa membrane serves as a mechanical and physical shield. The mechanical shield of the oral epithelium consists of stratified keratinocytes, which form a strengthened structure [1]. The physical shield of the oral epithelium also initiates an active immunological response by presenting antigens and producing cytokines and HDPs [2]. Several types of HDPs, including defensins, cathelicidins, and histatins, may have important roles in innate host defense. HDPs, which are mostly cationic and have amphipathic structures, provide non-specific and rapid defense against invading pathogens. In human saliva, histatins are the major HDPs that are constitutively produced and directly secreted by the submandibular, sublingual, and parotid glands. The salivary glands also secrete small amounts of defensins and cathelicidin; these peptides are also produced by neutrophils and oral epithelial cells. Certain types of defensins and cathelicidin are inducible by inflammatory cytokines, indicating that these peptides may be of crucial importance under inflammatory conditions [3,4]. All these peptides have a broad range of biological properties. In addition to antimicrobial, antifungal, and antiviral activities, some of these peptides also possess antitumor or immunomodulatory properties.

This review focuses on human cathelicidin in the oral cavity and discusses its importance and potential in the clinical therapy of oral diseases.

## The classification and the molecular structure of HDPs

HDPs are diverse in their sequence and structures. To date, almost 1000 naturally occurring HDPs from bacteria, fungi, plants, invertebrates, amphibians, and mammals have been described (<http://www.bbcm.univ.trieste.it/~tossi/amsdb.html>, and <http://aps.unmc.edu/AP/main.php>). They are generally amphipathic, small (12–50 amino acids), and have at least two positive charges (as arginine or lysine residues). The diversity of HDPs discovered is so great that it is only possible to categorize them broadly, on the basis of their secondary structure [5]. Basically, HDPs can be classified according to four characteristic structures [6]: (1)  $\beta$ -sheet

structures stabilized with two or three disulfide bonds such as mammalian defensins, (2) amphipathic  $\alpha$ -helical structures such as human cathelicidin (3) loop structures containing one disulfide bond such as dodecapeptide, and (4) extended structures such as PR-39 and histatins (Table 1) [7–30].

Three families of HDPs are expressed predominantly in humans: defensins, cathelicidin, and histatins. Defensins are small cysteine-rich HDPs that mainly form  $\beta$ -sheet structures stabilized by several (usually three) conserved intramolecular cysteine disulfide bonds and are typically 28–44 amino-acid residues. Three subfamilies,  $\alpha$ -,  $\beta$ -, and  $\theta$ -defensins, are expressed in vertebrates [9]. In humans,  $\theta$ -defensin mRNA is expressed; however, lack the corresponding peptides since the human  $\theta$ -defensin gene contains a stop codon in the signal sequence that aborts translation [31]. The insect and plant defensins contain six or eight cysteines in the disulfide

**Table 1** Structure-based classification of HDPs.

Representative peptides	Origin	Refs.
<b><math>\beta</math>-Sheet</b>		
$\alpha$ -Defensins [HNP1–4, HD5, HD6]	Human	[7–9]
$\beta$ -Defensins [HBD1–4]	Human	[10]
$\theta$ -Defensin	Rhesus monkeys	[11]
Tachyplesins	Horseshoe crab	[12]
*Protegrins	Pig	[13]
<b><math>\alpha</math>-Helical</b>		
*hCAP18/LL-37	Human	[14–16]
*BMAP-27,28	Bovine	[17]
Cecropins	Silk moth	[19,117]
Magainins	Frog	[20]
Dermaseptin	Frog	[21]
Buforin I, II	Toad	[22]
<b>Loop</b>		
*Dodecapeptide	Bovine	[23]
Thanatin	Hemipteran	[24]
Brevinins	Frog	[25,26]
<b>Extended</b>		
*Bac5, 7 (Pro- and Arg-rich)	Cow	[27]
*PR-39 (Pro- and Arg-rich)	Pig	[28]
*Indolicidin (Trp-rich)	Cow	[29]
Histatins (his-rich)	Human	[30]

Peptides derived from cathelicidins are indicated by asterisks.

bridge. In addition, insect defensins contain  $\alpha$ -helical disulfide bridges connected to a  $\beta$ -sheet structure stabilized by cysteine, which differs from vertebrate defensins [9]. Cathelicidins comprise a large number of precursors of HDPs that typically contain a conserved N-terminal sequence region that shares high homology with the proregion of cathelin, a cathepsin L inhibitor (hence the term cath-e-L-in). The C-terminal antimicrobial domain of different cathelicidin precursors varies widely in terms of sequence, composition, and structure. Processed cathelicidin peptides range from 12 to 80 or more amino acid residues in size and may have  $\beta$ -sheet,  $\alpha$ -helical, loop, or extended structures [32,33] (Table 1). The only human cathelicidin, antimicrobial peptide LL-37, is composed of 37 amino acid residues and a cysteine-free peptide that can adopt an amphipathic  $\alpha$ -helical conformation [34]. In contrast, histatins are family of small cationic histidine-rich peptides amounting to 3–5 kDa in the human saliva [35]. The major family members of histatins 1 and 3 are products of human genes alone: these are HIS1 and HIS2, respectively [36]. Histatin 5 originates from histatin 3 by post-translational processing. Histatin 1, 3, and 5 have linear extended structures containing 38, 32, and 24 amino acid residues, respectively, and the sequence of the first 22 amino acids of each histatin is identical [37].

### The molecular structure of human cathelicidin hCAP18/LL-37

Cathelicidins were first discovered in mammals but have been recently found in chickens and three species of fish (rainbow trout, Atlantic salmon, and hagfish). In particular, hagfish remarkably lacks essential components of adaptive immunity. The presence of cathelicidins in this very ancient species may indicate that cathelicidin genes developed early in vertebrate phylogeny [38].

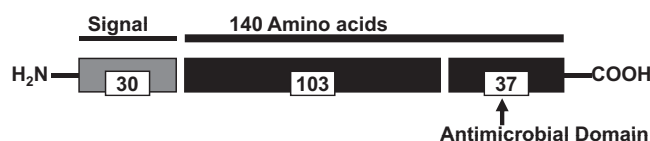
In humans, only one cathelicidin has been found from the myeloid bone marrow cDNA [14–16] and isolated from neutrophils [15]. In the human genome, cathelicidin exons 1–4 are found on chromosome 3p21. These are transcribed as a single gene, CAMP (cathelicidin antimicrobial peptide), which translates to an 18 kDa pre-pro-protein, referred to as hCAP18 [15,16]. The other term used to describe the protein is hCAP18/LL-37, because this protein is characterized by a N-terminal signal peptide (30 amino acid residues), a highly conserved prosequence (103 amino acid residues) called the cathelin like domain and a mature antimicrobial peptide named LL-37 (37 amino acid residues with Leu-Leu at the N-terminus) at the C-terminal domain [16] (Fig. 1). LL-37 has a net positive charge of +6 at the physiological pH, a hydrophobic N-terminal domain, and a  $\alpha$ -helical conformation most pronounced in the presence of negatively charged lipids [39]. LL-37 is produced from the C-terminal domain of the hCAP18/LL-37 precursor protein by proteolytic cleavage.

The hCAP18/LL-37 from specific granules of neutrophils is processed to active peptides LL-37 following exposure to the serine protease, as proteinase 3 from azurophilic granules after exocytosis. Proteinase 3 is cleaved at the hCAP18/LL-37 between the alanyl and leucyl residue [40]. However, proteinase 3 is expressed only myeloid cells and not epithelial cells. In recently study, the serine proteases stratum corneum tryptic enzyme (SCTE, kallikrein 5) and stratum corneum chymotryptic protease (SCCE, kallikrein 7) activates the precursor protein hCAP18/LL-37 on the skin surface [41]. In addition, the prostate-derived proteinase gastricsin (pepsin C) in the presence of vaginal fluid at low pH, can also process epididymal-derived hCAP18/LL-37 in seminal plasma to functionally active ALL-38 [42].

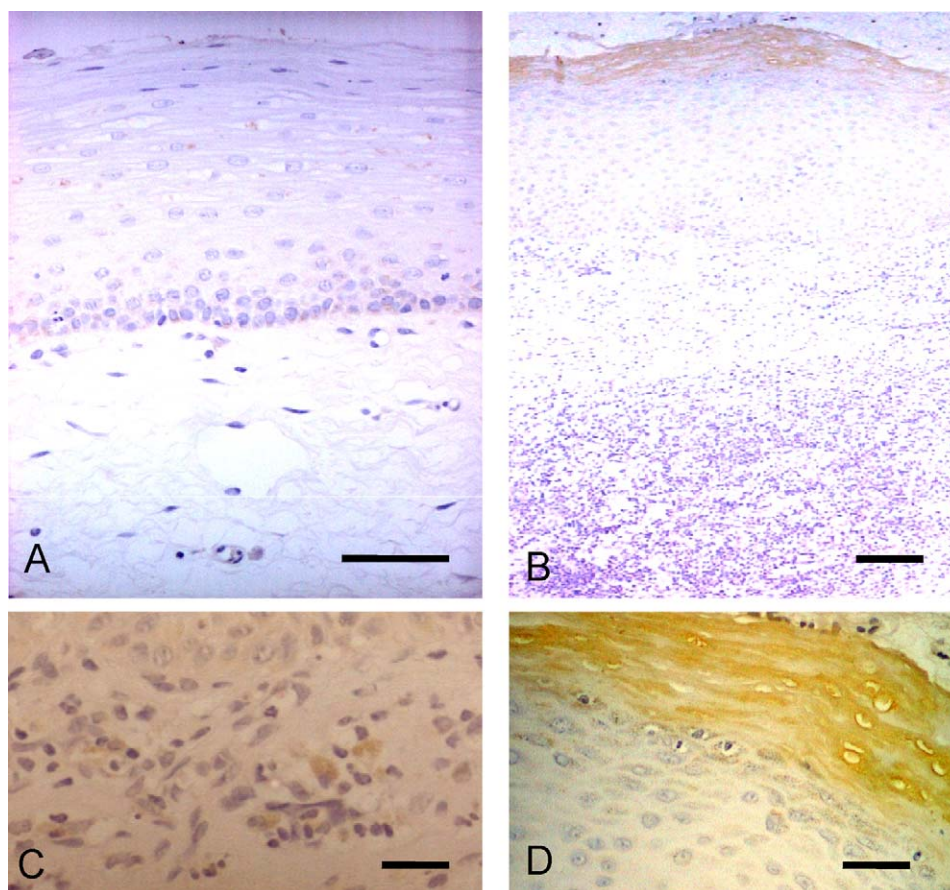
### Tissue distribution of hCAP18/LL-37

Most HDPs (bacterial/permeability increasing protein: BPI, azurocidin: CAP37, and  $\alpha$ -defensins) are localized in azurophilic granules [43–45]. In contrast, cathelicidin hCAP18/LL-37 is a major protein of the specific granules of immature neutrophils [46]. However, hCAP18/LL-37 shown to be produced in various blood cell populations, including NK cells,  $\gamma\delta$ T cells, B cells, monocytes [47], and mast cells by using RT-PCR, in situ hybridization, and immunohistochemical detection [48]. In addition, hCAP18/LL-37 is consistently expressed at both the mRNA and protein levels in the squamous epithelia of the airways, mouth, tongue, esophagus, intestine, cervix, and vagina. This peptide is widely produced in squamous epithelia; this suggests a role for this peptide in epithelial antimicrobial defense [49–51]. Furthermore, hCAP18/LL-37 was detected in the saliva and salivary glands, specifically in the acinar cells of the submandibular gland and palatine minor glands as well as in the lingual epithelium and palatal mucosa [52]. Additionally, it can be found in a number of other body fluids, including sweat, gastric juices, semen, plasma, airway surface fluid, and breast milk [53,54].

Epithelia not only provide a physical barrier between the body and the environment but also participate in the maintenance, renewal, and defense of these surfaces. Indeed, epithelia were found to be the second major producer of hCAP18/LL-37 after defensins [50,55]. In normal oral epithelium, hCAP18/LL-37 mRNA is strongly expressed in the basal layers and is decreased toward the surface, although its peptide immunoreactivity is also seen in the supra-basal layers [49] (Fig. 2(A)). The hCAP18/LL-37 mRNA and its protein, interestingly, are undetectable in normal skin [55,56]. One may argue that constitutive expression of its peptide may be more critical in epithelia lacking the outer keratinized cover in oral epithelial cells. The hCAP18/LL-37 is stored in secretory granules called the lamellar bodies of keratinocytes, as determined by immunogold electron microscopy [57].



**Figure 1** Schematic diagram of human CAP18 showing the signal peptide, N-terminal domain, and C-terminal LPS-binding domains.



**Figure 2** In noninflammatory and oral lichen planus of the gingival mucosa, hCAP18/LL-37 is expressed. (A) Noninflammatory gingival mucosa demonstrates positive immunoreactivity for hCAP18/LL-37 predominantly found in the basal epithelial layer. (B) Oral lichen planus demonstrates strong immunoreactivity for hCAP18/LL-37 in the superficial epithelial layer. (C) hCAP18/LL-37 immunoreactive stroma cells in the noninflammatory gingiva. (D) High-power view of superficial epithelial layer, same as in panel B. Bars: A and B, 100  $\mu$ m; C and D, 20  $\mu$ m.

## Various biological activities of the hCAP18/LL-37 peptide

### Mechanism of action and antimicrobial activity

HDPs-mediated microbial killing can be rapid: some linear  $\alpha$ -helical peptides kill microbes very quickly [6]. For example, cecropin P1 and PR-39 kill bacteria within 25 min [58]. Regardless of the specific antimicrobial mechanism, specific steps must occur in inducing bacterial death.

Antimicrobial activity occurs through several mechanisms. The first step in HDPs-mediated function is attraction. Attraction is considered to occur when the initial interaction between the cationic peptides first occur through electrostatic interactions with the negatively charged bacterial membrane. Interestingly, HDPs show significantly lower cytotoxicity to host cells because their membranes possess a high amount of cholesterol. The second step is attachment, where the peptides traverse the exterior capsular polysaccharides to reach the inner lipid layer. It is shown that two physiologically distinct states occur during this peptide-membrane interaction. At low peptide/lipid ratios,  $\beta$ -sheet (defensins) and  $\alpha$ -helical (LL-37) peptides first embed into the lipid head groups in a functionally inactive state, stretching the mem-

brane. At high peptide/lipid ratios, peptides orient perpendicularly and insert into the bilayer [59]. After insertion, antimicrobial peptides act via membrane permeation. Three main models of the action of membrane perturbation by HDPs have been proposed: the barrel-stave model, carpet model, and toroidal-pore model. In the barrel-stave model, peptide helices form a bundle in the membrane with a central lumen, very similar to a barrel, with the helical peptides as the staves. This model explains the activity of antimicrobial peptides such as the fungus antimicrobial peptide, alamethicin. In the carpet model, the peptides accumulate on the bilayer surface. They are electrostatically attracted to the anionic phospholipid head groups at numerous sites covering the surface of the membrane in a carpet-like manner. At high concentrations, the peptides are believed to disrupt the bilayer acting like a detergent, resulting in the formation of micelles. This type of transmembrane pore is induced by LL-37. The toroidal pore model combines the action of the two previous models and begins with aggregation on the membrane surface. The peptides insert into the membrane perpendicularly and induce continuous bending of the lipid monolayers through the pore lead to the water core to be lined by both the inserted peptides and the lipid head groups. During this action, the polar faces of the peptides associate with the polar head groups of the lipids, resulting in a



continuous bend that connects the two leaflets of the membrane. Thus, toroidal pore formations in the membrane result in the lipids forming micelles and subsequent membrane disruption. This model explains the activity of antimicrobial peptides such as magainins and protegrins [60].

Its antimicrobial properties led to the initial identification of hCAP18/LL-37 [39]. It exhibits antimicrobial activity against both gram-positive and gram-negative bacterial strains. The minimal inhibitory concentration (MIC) for LL-37 against these pathogens can range to less than 10  $\mu\text{g/ml}$  [34]. This peptide is active against clinically important strains of gram-negative bacteria and periodontal pathogens such as *A. actinomycetemcomitans* and *Capnocytophaga* [61]. Similarly, we showed that the LL-37-derived 27-mer synthetic peptide (hCAP18<sub>109–135</sub>) and its analogues (LL/CAP18 and FF/CAP18) killed *Porphyromonas* and *Prevotella* species within a short time and with a low peptide concentration [62] (Fig. 3). Additionally, LL-37 peptide is capable of killing gram-negative oral streptococci, including *Streptococcus mutans*, *S. sanguinis*, *S. salivarius*, and *S. mitis*. The LL-37 is particularly effective in killing these streptococci, especially *S. mutans*, when they act as cariogenic pathogens [63]. Our study demonstrated that the LL-37-derived synthetic peptides exerted potent antimicrobial activity against *Streptococcus sanguis* isolated from patients with Behçet's

disease (BD), thereby producing a stronger killing activity [64] (Fig. 4). Hence, LL-37 is related to oral mucosal defense, and the regulated expression and production of this peptide can be important for the suppression of BD. Furthermore, this antimicrobial activity is augmented by  $\alpha$ - or  $\beta$ -defensins in vitro [65]. These data suggest that the LL-37 peptide acts synergistically under in vivo conditions to form an efficient barrier against microbial invasion.

In the oral cavity, microbes are exposed to saliva and serum, which contain salt and reduce the antimicrobial activity of  $\beta$ -defensins by 50% of the activity observed under control (salt-free) conditions [63]. In contrast, cathelicidin LL-37 are active against several bacteria in high salt media [34,65,66], supporting its capacity to function under a variety of physiological conditions. Additionally, high ionic concentrations are generally found in body fluids, including the saliva of patients with cystic fibrosis [67]. In a cystic fibrosis xenograft model, gene transfer of hCAP18/LL-37 restored bacterial killing to normal levels [68]. This report suggests that hCAP18/LL-37 may confer protection against bacterial infections in vivo.

In *Candida albicans*, LL-37 can disrupt the cell wall and the cell membrane. Thus, peptide-induced membrane permeabilization increases the inhibition of *C. albicans* growth [69–71].

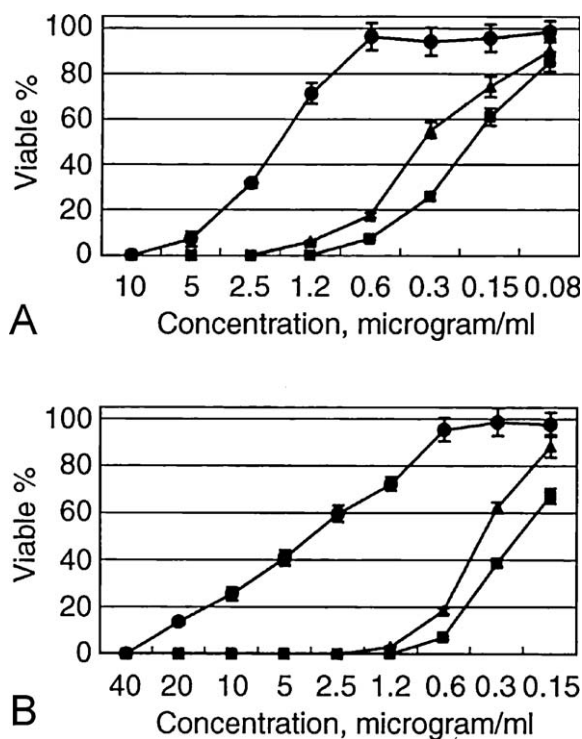
HDPs are known to contain some antiviral activity. For example,  $\beta$ -sheet peptides such as defensins, tachyplesin, and protegrins provoked remarkable inactivation of HSV [72]. Furthermore,  $\alpha$ -helical peptide as LL-37 inhibits virus replication against vaccinia (smallpox) virus [73]. In addition, LL-37 exhibits antiviral activity against HSV-1 in corneal and conjunctival epithelia [74].

### Peptide interaction with tumor cells and antitumor activity

Existing chemotherapeutic drugs that are widely used in cancer treatment have the severe side effect of nonspecific cytotoxicity. These agents target any rapidly dividing cells, without discriminating between healthy and cancerous cells. Furthermore, many cancers eventually become resistant to conventional chemotherapy through selection for multidrug-resistant variants [75]. Thus, there is an urgent need to develop new antitumor drugs with new modes of action that selectively target the cancerous cells.

Most HDPs have a cationic amphipathic structure, and they preferentially bind and insert into the negatively charged surfaces of bacterial cell membranes. The consequent destabilization of the membranes disturbs electrolyte balance and causes leakage of the intercellular contents, leading to cell death. Normal mammalian cell membranes generally have a neutral net charge, and their membranes are enriched in phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM), and cholesterol. In contrast, bacterial cell membranes are negatively charged with higher proportions of phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidylserine (PS), and have lower cholesterol content [76]. Thus, differences between the host and bacterial cell membranes exist, and these present potentially selective targets for HDPs.

Several HDPs preferentially disrupt bacterial and cancer cell membranes rather than host eukaryotic cell membranes



**Figure 3** Killing of *Porphyromonas* in the presence of various concentrations of hCAP18-derived peptides. hCAP18-derived peptides demonstrated antimicrobial activity against *P. gingivalis* 381 (A). hCAP18-derived peptides also demonstrated antimicrobial activity against *P. circumdentaria* NCTC12469 (B). Incubated for 15 min in HBSS (pH 7.4) for 37 °C. The points and error bars are expressed as the mean and standard deviation of three independent assays. ● hCAP18<sub>109–135</sub>, ▲ LL/CAP18, and ■ FF/CAP18 (see ref. [44]).

Bacterial strain	IC <sub>50</sub> (μg/ml)		
	hCAP18 <sub>109-135</sub>	LL/CAP18	FF/CAP18
<i>S. sanguis</i> BD113-20	1.0	0.15	<0.15
<i>S. sanguis</i> BD114-23	3.3	2.5	<0.15
<i>S. sanguis</i> BD118-1	4.8	2.1	0.24
<i>S. sanguis</i> SSH-83	4.2	0.65	0.95
<i>S. sanguis</i> KTH-T	8.4	2.2	<0.15
<i>S. sanguis</i> ATCC10556	7.0	0.18	0.46
<i>S. oralis</i> ATCC10557	4.8	2.5	1.12
<i>S. gordonii</i> ATCC10558	0.53	0.21	0.52
<i>S. gordonii</i> ST-7	3.7	0.11	<0.15

**Figure 4** Antimicrobial activity of hCAP18-derived peptides—IC<sub>50</sub>. The IC<sub>50</sub> shows that hCAP18 is active against all strains at concentrations ranging from 0.5 to 8.4 μg/ml. The IC<sub>50</sub> of the CAP18 analog peptide (LL/CAP18 or FF/CAP18) is showed a maximum 10-fold stronger potential than that of hCAP18<sub>109-135</sub> (see ref. [45]).

[77,78]. The cancer cell membranes contain a large amount of negatively charged PS, which is more negative than that of normal eukaryotic cells [79]. Therefore, it has been suggested that the increase in negatively charged PS in the cancer cell membranes makes them more susceptible to the cytotoxicity of the peptides than normal eukaryotic cells [80]. In addition, these peptides that disrupt target cell membranes as part of their killing effect show irreversible activity [81,82].

The existence of antitumor activity was first proven in a study of the antimicrobial peptide magainin (a non-hemolytic α-helical peptide) and its synthetic analogues, which are active against hematopoietic and solid tumors at concentrations that are moderately nontoxic to normal cells [83]. Similarly, HDPs act via a non-receptor-mediated pathway against the target cell membranes as do melittin (a non-selective cytotoxic α-helical peptide), cecropin (a non-hemolytic α-helical peptide), and androctonin (a non-hemolytic β-sheeted peptide) [84,85]. Therefore, the biological activity shown by these peptides suggests the existence of killing mechanisms that involve perturbation of the plasma membrane, inducing necrosis. Membrane-active peptides can induce the permeabilization of mitochondria, triggering apoptosis [82]. A cationic membrane-active antimicrobial peptide, CNGRC-GG-D(KLAKLAK)<sub>2</sub>, shows antitumor activity via the mitochondrial pathway of apoptosis [86]. Similarly, tachyplesin, a heptadecameric cationic antimicrobial peptide, could interact with the mitochondrial membranes of cancer cells and induce apoptosis [87,88]. Thus, the cationic antimicrobial peptides exhibit antitumor activity. In the cathelicidin family, the bovine produced BMAP-27 and BMAP-28 have been shown to induce membrane permeabilization and apoptosis in human leukemic tumor cells and normal proliferating but not resting lymphocytes [89,90]. This is associated with cathelicidin peptide-induced membrane permeabilization and is followed by programmed cell death. This indicates that BMAP-28 induced mitochondrial membrane permeability and then caused mitochondrial depolarization and released cytochrome c, leading to apoptosis [90].

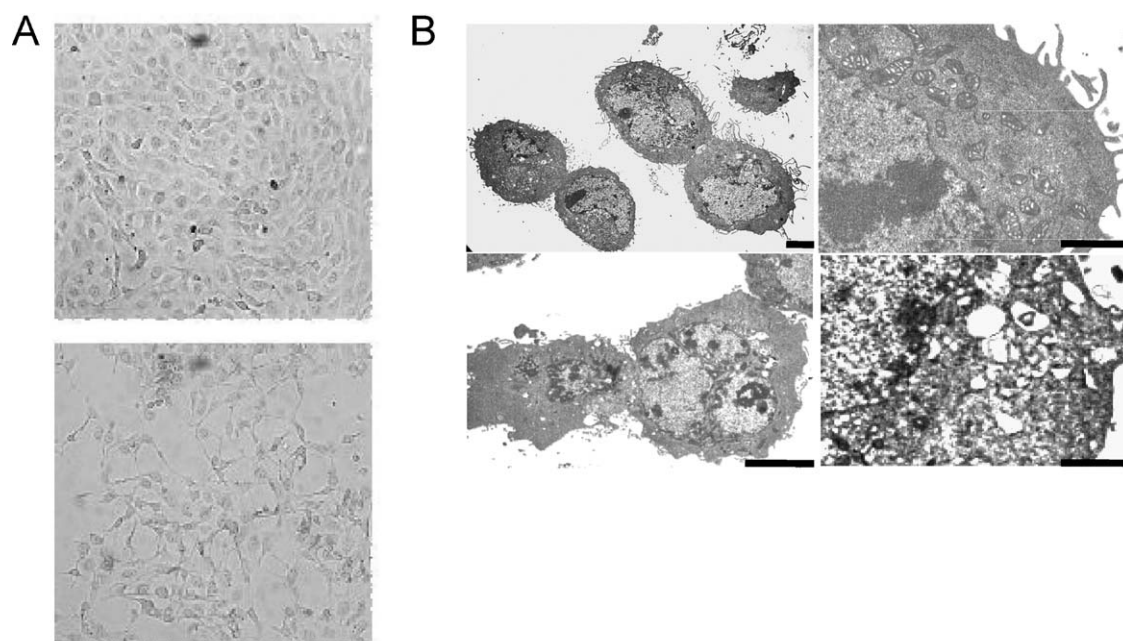
We have previously synthesized a 27-amino acid sequence from the C-terminal domain of hCAP18/LL-37 and described its antimicrobial activity against *Porphyromonas* and *Prevotella* species [62]. This peptide is designated hCAP18<sub>109-135</sub> and consists of the active domain, as LL-37. In our recent study, hCAP18<sub>109-135</sub> induced apoptotic cell death of squa-

mous cell carcinoma cells, but not gingival fibroblasts or normal keratinocytes and HaCaT cells [91]. The hCAP18<sub>109-135</sub> induced apoptotic cell death was attributed to a caspase-independent pathway (Fig. 5, data unpublished). Furthermore, we demonstrated a correlation between different apoptotic events affecting the mitochondria, cytosol, and nuclei following hCAP18<sub>109-135</sub> inductions. In order to examine the apoptotic effect of hCAP18<sub>109-135</sub> on human squamous cell carcinoma SAS-H1 cells, the peptide was added at a concentration of 40 μg/ml in the presence of 10% fetal bovine serum. We showed that hCAP18<sub>109-135</sub> elicited the translocation of Bax to the mitochondria and endonuclease G to the cytosol. Thus, in peptide-induced cell death, Bax-dependent endonuclease G release plays a role in caspase-independent oligonucleosomal DNA fragmentation (Figs. 6 and 7, data unpublished). The active domain peptide LL-37 of hCAP18/LL-37 is also shown to induce the apoptosis of human lung carcinoma A549 cells, SV40-transformed, immortalized 16HBE4o-human airway epithelial cells, and primary human bronchial epithelial cells [92,93]. However, in contrast to our observations, the LL-37 induced apoptosis via caspase-3 activation; these data indicate caspase-dependent programmed cell death. Importantly, this peptide suppressed the apoptosis of neutrophils [94]. In this context, the mechanisms involved in the apoptotic and antiapoptotic actions of these peptides remain to be determined.

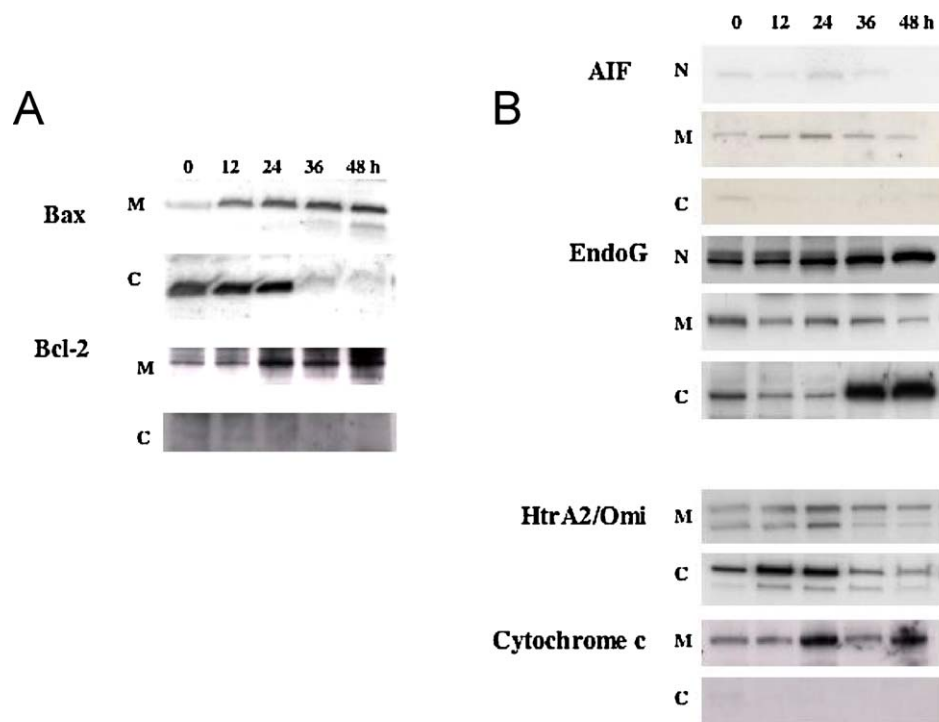
Taken together, these studies show that the mechanism of tumor cell killing by host defense peptides is poorly understood. However, in particular, human cathelicidin peptides have selective cytotoxicity toward tumor cells and may be useful antitumor therapeutic agents.

### Other biological activities

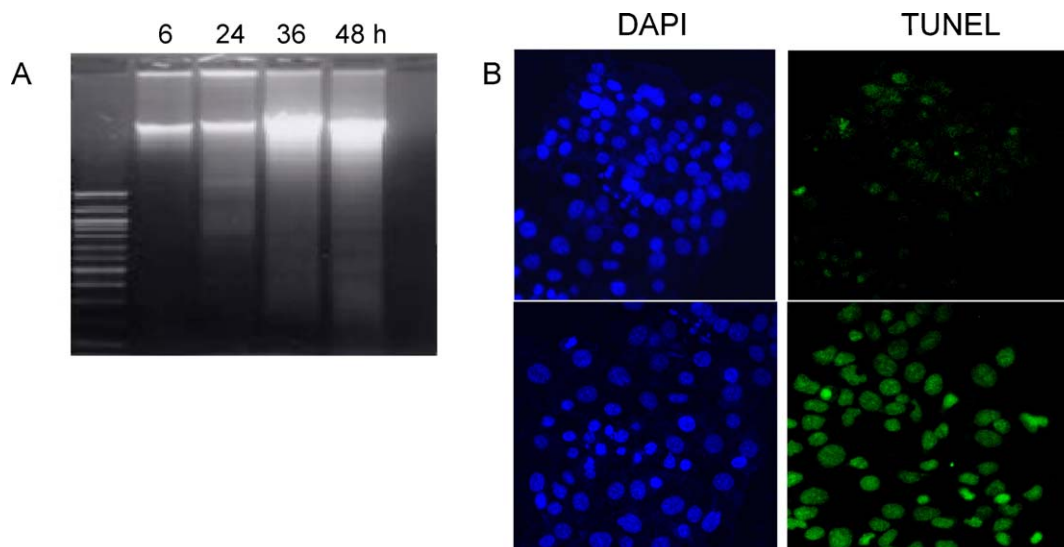
A previous study showed that LL-37 protects against endotoxin shock [95]. Lipopolysaccharide (LPS) is a cell membrane component of gram-negative bacteria. LPS has strong biological activity and plays a key role in the pathogenesis of endotoxin shock associated with various syndromes [96,97]. LPS induces monocytes, macrophages, and other types of cells to produce and release potent pro-inflammatory cytokines. However, LL-37 can neutralize the biological activity of LPS by binding to it with higher affinity [98–100]. Indeed, we found that the LL-37-derived 27-mer synthetic peptide (hCAP18<sub>109-135</sub>) suppressed development of endotoxin-induced uveitis in vivo in rats



**Figure 5** hCAP18-derived peptide induced morphological alteration in tumor cells. The SCC cells, SAS-H1 cells, were cultured in the absence (top) or presence (bottom) of 40  $\mu\text{g/ml}$  hCAP18<sub>109–135</sub> for 24 h and then photographed using digital image capture and a phase-contrast light microscope (A). Transmission electron microscopic micrographs showing differences in SAS-H1 cells and mitochondria morphology upon hCAP18<sub>109–135</sub> exposure. Untreated SAS-H1 cells and mitochondria (top) versus hCAP18<sub>109–135</sub>-treated SAS-H1 cells and mitochondria (bottom) showing severe ultrastructural changes such as disorganization and swelling of the mitochondrial organelle. Bars: 1  $\mu\text{m}$  (B).



**Figure 6** hCAP18-derived peptide induces alterations in the Bax/Bcl-2 ratio and mitochondrial release proteins of SAS-H1 cells. SAS-H1 cells were treated with 40  $\mu\text{g/ml}$  hCAP18<sub>109–135</sub> for the indicated time points. M: mitochondrial fraction, C: cytosolic fraction. Equal amounts of cell lysates were separated by SDS-PAGE, followed by immunoblotting with anti Bax and anti Bcl-2 antibodies (A). Cells were treated for the indicated time points with hCAP18<sub>109–135</sub>. N: nuclear fraction, M: mitochondrial fraction, and C: cytosolic fraction were analyzed by immunoblotting for the presence of AIF, Endo G, HtrA2/Omi, and cytochrome c (B).



**Figure 7** hCAP18-derived peptide induces apoptosis in SAS-H1 cells. Agarose gel electrophoresis analysis of DNA fragmentation in SAS-H1 cells following treatment with complete medium alone or in the presence of 40 µg/ml hCAP18<sub>109–135</sub> for 6, 24, 36, and 48 h (A). TUNEL assay on peptide-treated SAS-H1 cells. SAS-H1 cells were cultured in the absence (top) or presence (bottom) of 40 µg/ml hCAP18<sub>109–135</sub> for 48 h and then observed using confocal laser microscopy. DAPI: nucleic acid staining (B).

[101]. Therefore, the hCAP18<sub>109–135</sub> suppresses the onset of LPS-triggered inflammatory reactions by binding directly to LPS.

LL-37 demonstrates chemotactic activity for T lymphocyte cells, monocytes, and neutrophils [102], thus attracting even more leukocytes to the site of increased LL-37 concentration in inflamed or infected tissue. In addition, mast cells, which form an important tissue-localized part of innate immunity, exhibited LL-37-induced migration, histamine release, and intracellular Ca<sup>2+</sup> mobilization [103]. LL-37 was proven to activate at least three different receptors, namely FPRL-1 (formyl peptide receptor-like 1) [102,103], EGFR (epidermal growth factor receptor) [104], and the purinergic receptor P2X<sub>7</sub> [105]. Furthermore, a vitamin D responsive element is identified in the human cathelicidin gene CAMP promoter region [106]. The vitamin D3 signaling cascade that leads to increased cathelicidin expression has been identified [107,108]. LL-37 modulates dendritic cell (DC) differentiation and enhances the secretion of helper T cell type 1 (Th-1)-inducing cytokines in vitro [109]. These results suggest that hCAP18/LL-37 bridges the innate and adaptive immune responses. Recently, the hCAP18/LL-37 peptide has been identified as the key factor that mediates the activation of plasmacytoid dendritic cells (pDCs) in psoriasis, a common autoimmune skin disease. pDCs do not normally respond to self-DNA, but binding to hCAP18/LL-37 converted DNA into a potent stimulus for pDC activation. The hCAP18/LL-37 and self-DNA complexes signaled through TLR9 and elicited interferon (IFN)-α release from pDCs. IFN-α subsequently activated a T-cell response that could lead to inflammation [110]. Interestingly, pDCs are also found in oral lichen planus (OLP) and in periodontitis [111,112]. In particular, significant recruitment of pDCs producing IFN-α within the lichenoid inflammatory infiltrate and close cell–cell contacts between pDCs and mature dendritic cells (DCs) have been demonstrated. These data indicate that recruitment of different subtypes of DC, including pDCs, may play a pivotal role in the development of the lichenoid inflammatory infil-

trate that typically occurs in OLP. We hypothesize that by analogy with the hCAP18/LL-37 and self-DNA complexes that activate pDCs in psoriasis, its peptide increases in OLP. Binding of self-DNA released from damaged or apoptotic cells to hCAP18/LL-37 may be potentially developed as therapies for OLP and other chronic inflammatory diseases.

Additionally, hCAP18/LL-37 induced angiogenesis, and its peptide resulted in neovascularization both in the chorioallantoic membrane assay and in a rabbit hind-limb ischemia [113]. This peptide directly activated endothelial cells to proliferate and form vessel-like structures in human endothelial cells, HUVECs, and shown to cause endothelial sprouting from hamster aortic rings. The angiogenic activity of hCAP18/LL-37 appears to be mediated by the interaction of peptide with FPRL-1 in endothelial cells.

## The involvement of hCAP18/LL-37 in the oral diseases

### Caries and periodontitis

It has been demonstrated that human β-defensin-3 (hBD-3), hCAP18/LL-37, and α-defensins are present in the µg/ml range in children's saliva. The concentration of α-defensins was significantly higher in children with no caries than in those with caries, whereas the concentration of hCAP18/LL-37 and hBD-3 did not correlate with caries [114]. In contrast, the expression of hCAP18/LL-37 was upregulated in the inflamed gingival tissue in comparison with healthy gingival tissue and was correlated positively with the depth of the gingival crevice [115], indicating that hCAP18/LL-37 expression in the gingival tissue is associated with the severity of periodontal disease. In addition, there are variations in the responses of oral epithelial cells to different bacteria and in the sensitivity of oral flora to the peptides [116]. For example, *Prevotella intermedia* induced the expression of hCAP18/LL-37 and hBD-1, hBD-2, and hBD-3; *Fusobacterium*



*nucleatum*, hBD-2 and hBD-3; and *Porphyromonas gingivalis*, hBD-2. Other species associated with periodontal disease, such as *Tannerella forsythia* and *Treponema denticola*, either did not induce expression or caused a down-regulation of steady state mRNA levels. Expression of hCAP18/LL-37 in the gingival epithelial cells was similar; *P. gingivalis* did not induce expression, whereas *A. actinomycetemcomitans*, *F. nucleatum*, *P. intermedia*, and *E. corrodens* upregulated the expression of hCAP18/LL-37 [115].

### Periodontitis associated with congenital diseases

In severe congenital neutropenia (SCN, or morbus Kostman) was associated with hCAP18/LL-37 deficiency, and the reduced levels of  $\alpha$ -defensins (HNP1-3) were also found in the neutrophils of patients with SCN. Furthermore, hCAP18/LL-37 is completely absent from the plasma and saliva of these patients; consequently, these patients present chronic periodontitis and overgrowth of *Actinobacillus actinomycetemcomitans*. Surprisingly, hCAP18/LL-37 has been reported to have antimicrobial activity against *A. actinomycetemcomitans*, supporting the hypothesis that the deficiency of its peptide may result in periodontitis. In addition, despite normalized absolute neutrophil count levels, G-CSF-treated SCN patients still often have periodontitis [117,118]. According to the maturation cycle of neutrophils, defensins are predominantly detected at the promyelocyte stage, when primary granules mature. In contrast to hCAP18/LL-37, which is primarily detected at the myelocyte stage, when secondary granules mature [119]. Recently, HAX1 gene mutations in SCN patients that result in increased apoptosis in myeloid cells have been identified. The HAX1 gene encodes the hematopoietic cell-specific protein 1 (HS1)-associate protein X-1 (HAX-1), which has been regulated in apoptosis [120]. Thus, deficiency of hCAP18/LL-37 may be associated with maturation arrest in myelopoiesis. Similarly, severe periodontitis is found in the Papillon–Lefèvre syndrome (PLS), an inheritable disease caused by loss-of-function mutations in the cathepsin C gene. Cathepsin C is the activator of serine proteinases, elastase, cathepsin G, and proteinase 3. These patients have been recently found to lack active neutrophil-derived serine proteases. The neutrophils of PLS patients release reduced levels of mature hCAP18/LL-37 because serine proteinases are needed to convert the neutrophil-derived hCAP18/LL-37 into the mature peptide that possesses antimicrobial activity [121].

These studies suggest that hCAP18/LL-37 plays an important role in innate immunity against periodontal pathogens.

### Inflammatory epithelial diseases

In keratinized epithelial cells, hCAP18/LL-37 is inducible with inflammatory disorders, psoriasis, and nickel allergy [50]. Under inflammatory conditions, the epidermis showed abundant immunohistochemical staining of its peptide, while the healthy dermis did not show the presence of the peptide. In non-keratinized epithelial cells, under conditions of dysplasia and inflammatory cervix of the uterus, the strongest expression of hCAP18/LL-37 was detected at both the mRNA and protein levels in the upper spinous and granular layers toward the surface [49]. In fact, we found that the oral lichen

planus (OLP) expresses more intense immunohistochemical staining for the hCAP18/LL-37 peptide than the normal epithelium (Fig. 2, unpublished data). Similarly, OLP showed intense immunohistochemical staining of  $\beta$ -defensin-2 (hBD-2) [122]. This increased expression is not related to microbial infection. For example, hCAP18/LL-37 mRNA and its peptide are rapidly expressed in the skin at the site of injury. Moreover, expression of hCAP18/LL-37 in the keratinocytes occurred in response to a sterile surgical incision [56]. The growth factors important in wound healing, such as insulin-like growth factor I (IGF-I) and transforming growth factor- $\alpha$  (TGF- $\alpha$ ), induce the expression of hCAP18/LL-37 and  $\beta$ -defensin-3 (hBD-3), respectively, in human keratinocytes [123]. In addition, IGF-I and TGF- $\alpha$  expression is increased in the psoriatic epidermis and during wound healing [124–127]. The generation of these growth factors in inflamed lesions without microbial infection may have contributed to this response. In contrast, in cases of inflammatory disease, atopic dermatitis shows significantly lower expression of hCAP18/LL-37 and hBD-2 than psoriasis [128]. Similarly, hCAP18/LL-37 expression has been shown to decrease in the chronic ulcer epithelium [129]. Decreased expression of these peptides can explain the increased susceptibility to microbial colonization and infection. Consequently, the level of hCAP18/LL-37 expression may be related to the causes of a disease. For instance, at low concentrations of hCAP18/LL-37 found in normal human epithelia, its peptide could function as an immune watchdog and in cases of high concentrations, when hCAP18/LL-37 is induced by bacteria, bacterial products, or inflammatory cytokines, its peptide could function to promote the migration of immune cells to help control the infection.

### Malignant tumors

Alterations in hBD expression in oral squamous cell carcinoma (SCC) have been shown. The expression levels of hBD-1 and hBD-2 varied among cell lines derived from human oral SCC. Although high hBD-2 mRNA expression is detected in all cell lines examined, some of the cell lines did not show hBD-1 mRNA expression [130]. In addition, hBD-1 and hBD-2 mRNA expression was significantly lower in oral SCC as compared to the normal oral epithelium [131]. An immunohistochemical study indicated that well-differentiated SCCs showed strong immunoreactivity for hBD-2 around keratin pearls, whereas no immunoreactivity was observed in poorly differentiated SCCs. Keratin pearl formation with SCC tends to induce a more intense expression of hBD-2 at both peptide and mRNA levels. In contrast, hBD-2 peptide and its mRNA are undetected in poorly differentiated SCCs [132]. Similar results are obtained in cervical cancer [133]. In general, poorly differentiated SCCs have a more aggressive course and worse prognosis than well-differentiated SCCs. These results suggest that hBD deficiency leads to the formation of microbial colonies, which in turn induces inflammation and promotes tumor progression.

We have demonstrated that the expression of hCAP18/LL-37 mRNA is undetected in 16 cell lines from human oral SCC (data unpublished). A similar result is obtained in that hCAP18/LL-37 peptide expression is decreased in colonic epithelial cancer cells than in the normal colonic tissue. In addition, it is demonstrated by using an in vitro model of

colon epithelial cell differentiation that the expression of hCAP18/LL-37 mRNA and its protein increase during differentiation [51]. These results indicate that cell differentiation may be a determinant for the upregulation of epithelial hCAP18/LL-37 expression.

Conversely, hCAP18/LL-37 is highly expressed in human breast cancer cells with a correlation between its peptide protein levels and tumor grade than normal mammary tissue [134]. In addition, hCAP18/LL-37 is highly expressed in breast cancer, correlating with the expression of the ERBB2 gene; its peptide amplifies mitogen-activated protein kinase (MAPK) signaling through ErbB2 and treatment with the LL-37 peptide stimulates migration of cancer cells [135]. Similarly, hCAP18/LL-37 is significantly overexpressed in ovarian cancer cells; its peptide induces ovarian cancer cell proliferation, migration, invasion, and matrix metalloproteinase (MMP) activation through formyl peptide receptor-like 1 (FPRL1) signaling [136,137]. Furthermore, the hCAP18/LL-37 peptide is expressed mostly in human lung cancers. Overexpression of hCAP18/LL-37 in lung cancer xenografts increases the formation of significantly larger tumors in nude mice [138]. Collectively, these results suggest that hCAP18/LL-37 is an autocrine survival factor released by cancer cells.

However, the involvement of hCAP18/LL-37 in human OSCC remains to be elucidated. Further study is needed to clearly understand this phenomenon.

### Therapeutic potential of HDPs (cathelicidin-based agents)

HDPs exhibit broad-range antimicrobial activity and a low probability of resistance development [139]. They represent ideal potential therapeutics. However, several issues stand in the way of their development; the difficulty and high cost of manufacturing peptides is arguably the principal problem preventing the widespread clinical use of this class of antimicrobial therapeutics [140].

In the oral cavity, HDPs, including the hCAP18/LL-37 peptide, play important roles in maintaining oral health. Therapeutic use of HDPs in oral care requires clinical studies with defined end points due to the complexity of the etiology and pathogenesis of oral complications. Human trials failed to support the use of isegenan (protegrin variant), as cathelicidin family peptide to reduce the severity of oral mucositis [141], although microbial limit testing and safety studies clearly indicated the efficacy of histatin in animals [142,143]. In addition, adsorption of histatin 5 onto a poly (methyl methacrylate) denture base can prevent *C. albicans* biofilm formation, thus serving to reduce denture-induced stomatitis [144]. Recently, it was shown that hCAP18/LL-37 potentially inhibited the formation of *Pseudomonas aeruginosa* biofilms in vitro. This occurred at a very low and physiologically meaningful concentration of 0.5 µg/ml, far below that required to kill or inhibit growth [145].

Although lactoferrin (LF), an iron-binding glycoprotein, is originally identified as an HDP, in addition to antimicrobial activity and immunomodulatory functions, it also displays antitumor activity. LF has been shown to have anti-cancer activity in various malignant tumors [146]. For example, talactoferrin (TLF), a recombinant human lactoferrin, is an immunomodulatory molecule that has shown promising anti-

tumor activity in preclinical models and in a variety of solid tumors. Phase II trial data suggested that TLF is a promising, well-tolerated agent that has demonstrated evidence of potential clinical activity in metastatic renal cell carcinoma [147].

Cationic antimicrobial peptides, including human cathelicidin hCAP18/LL-37, possess qualities that make them excellent candidates for antimicrobial therapeutics, including a broad spectrum of antimicrobial activity, ease of synthesis, and a novel mechanism of action. In a recent report, it was shown that in bacterial infections with *Shigella*, expression of hCAP18/LL-37 and hBD-1 is reduced or turned off, which could partly explain the chronic inflammatory response associated with *Shigella* infection. Remarkably, the study further demonstrated that plasmid DNA released from lysed bacteria by the action of hCAP18/LL-37 was a major mediator of antimicrobial peptide down-regulation [148]. Therefore, hCAP18/LL-37 can act as a nuclear localization signal to translocate antisense nucleic acids [149]. Interestingly, a recent study demonstrated that treatment with a combination of CpG oligodeoxynucleotides (CpG-ODN), broadly as immunostimulant, and LL-37 generated significantly better antitumor therapeutic effects and enhanced survival in murine ovarian tumor-bearing mice than treatment with CpG-ODN or LL-37 alone [150]. The expression of CD69 and IFN-γ in NK cells stimulated by CpG-ODNs can be enhanced by treatment with the LL-37 peptide, thus leading to the activation of NK cells. NK cells play a critical role in the antitumor effects against murine ovarian tumor.

Although HDPs, including hCAP18/LL-37, indicate antimicrobial and antitumor activity, it is currently difficult to develop peptide-based drugs due to poor pharmacokinetics and potential systemic toxicity [151].

### Conclusion

Cathelicidins are an important family of HDPs because they are multifunctional, and their significance in human immune defenses is only beginning to be fully recognized. Additionally, hCAP18/LL-37 elicits complex responses in many cell types, either directly or through the modulation of cellular responses to microbial compounds and other immune mediators. Their HDPs may be useful in the diagnosis and therapy of periodontal and cariogenic diseases and in oral mucositis. Further advances in our understanding of the biological activity of HDPs, including hCAP18/LL-37, will be of therapeutic potential in infectious, inflammatory, and cancerous diseases.

### Conflicts of interest

No potential conflicts of interest were disclosed.

### Acknowledgments

I am grateful to E. Isogai from the Laboratory of Animal Microbiology, Graduate School of Agricultural Science, Tohoku University, for the critical reading of the manuscript and to T. Shibata from the Division of Reconstructive Surgery for Oral and Maxillofacial Region, Department of Human

Biology and Pathophysiology, School of Dentistry, Health Sciences University of Hokkaido, for the help with preparing the manuscript. The work conducted in the author's laboratory was supported by a Grant-in-Aid for Scientific Research on Priority Areas (C) (2), #19592309 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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